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TITLE: Development of novel non-against PPAR-gamma ligands for lung cancer treatment.

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14. ABSTRACT The overall goal of this grant is to establish the role of non-agonist PPAR-gamma ligands as potential therapeutic candidates for lung cancer. In this grant period, we have built on our gene expression data to show that a PPAR-gamma S273 phosphorylation signature is correlated with response to chemotherapy in lung cancer in publicly available datasets. We have further shown genetically using lung cancer cell lines lacking p53, that p53 is an important mediator of ability of non-agonist PPAR-gamma ligands to sensitize lung cancer to DNA damaging agents. We have demonstrated a biochemical interaction between p53 and PPAR-gamma, which provides insight into the groups of patients for whom this combination therapy may benefit. We have also found new interactions of PPAR-gamma with other players in DNA damage repair, including BRCA1 and gamma-H2AX. We continue to make progress on the other aims of this grant, which aim to test this hypothesis in genetic animal models of lung cancer and to identify novel partners for PPAR-gamma that may play a role in DNA repair.						
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INTRODUCTION

In the veteran population, lung cancer is the number one cause of cancer death. Our previous work demonstrated that ligands for the transcription factor PPAR γ , the thiazolidinediones (TZDs), synergized with carboplatin treatment of lung cancer in vitro and in vivo. Unfortunately, the use of TZDs has declined as the adverse effects of these drugs have come to light. Recently, we have demonstrated that novel PPAR γ ligands that lack any agonist activity, but inhibit phosphorylation of pS273 are effective anti-diabetic agents that lack many side effects caused by TZD treatment. To generate novel therapeutics with potential in lung cancer, we have explored the role of these non-agonist PPAR γ ligands in cancer treatment. We have demonstrated that there is robust phosphorylation of PPAR γ after carboplatin treatment in A549 cells, which can be inhibited by non-agonist ligands (NALs). These drugs are active in vitro and in vivo in genetic mouse models and xenografts. In this work, we are exploring the role of PPAR γ ligands in lung cancer treatment by identifying biomarkers of activity, mechanisms of action and validating their role in genetic models of lung cancer. We have generated a gene set representative of the activity of these agonist ligands in combination with carboplatin. We have identified p53 signaling as a key mediator of the ability of these non-agonist ligands ability to sensitize cells to carboplatin. Given the broad importance of this pathway in the DNA damage response, we have also shown that non-agonist PPAR γ ligands are able to sensitize lung cancer to a wide variety of DNA damaging agents, which further broadens the potential clinical applications of this line of inquiry. We anticipate this will develop a new avenue to combine anti-diabetic drugs and cancer therapy and will lead to a significant improvement in overcoming treatment resistance or chemoprevention of lung cancer death for veterans

KEYWORDS

PPAR-gamma; Lung cancer; DNA damage response; Thiazolidinediones; p53

ACCOMPLISHMENTS

Aim 1 / Major Task 1: Generate gene set altered by carboplatin treatment with and without inhibition of pS273 phosphorylation.

One of the goals of the project was to generate a gene set representative of the inhibition of pS273 phosphorylation after carboplatin treatment. My previous report details the generation of the PPAR γ S273A gene set and validation. In the period covered in this report, we have investigated whether this gene set can give insight into the mechanism of action of PPAR γ NALs.

As described previously, the gene set enrichment analysis and our immunoprecipitation experiments from Aim 2 have implicated the p53 pathway as a critical parameter involved in the response of cancer cells to NALs. We examined the effects of a non-agonist PPAR γ ligand in combination with carboplatin in Calu-1 cells, which have a genetic deletion of p53 (Appendix Figure 1A lanes 1-6,) as well as H2009 cells, which express mutant p53 (Appendix Figure 1B). In both of these cell types, we fail to see an increase in the DNA damage marker γ -H2AX when cells are treated with both SR1664 and carboplatin. To demonstrate that this lack of sensitization was due to the lack of p53, we ectopically expressed p53 in these cells by transient transfection.

Although the H2009 cells died upon introduction of wild type p53, Calu-1 cells expressing wild type p53 showed increased γ -H2AX accumulation when treated with SR1664 and carboplatin (Appendix Figure 1A, lanes 7-12.) These data suggest that the presence of wild type p53 is required for the sensitizing effects of non-agonist PPAR γ ligands.

To further investigate the role of p53 in the ability of non-agonist ligands to sensitize cells to DNA damaging agents, we performed Crispr/Cas9 mediated deletion of TP53 (Appendix Figure 1C) from A549 cells. Control cells transduced with Cas9 alone show robust increases in cleaved PARP and cleaved Caspase 3 when treated with the non-agonist ligand SR10171 and doxorubicin compared to doxorubicin alone (Appendix Figure 1C lane 3 vs. lane 6.) Contrastingly, cells depleted of p53 show no significant increase in accumulation of apoptotic markers when co-treated with SR10171 and doxorubicin compared to doxorubicin. Additionally, these cells show no increase in γ -H2AX phosphorylation when co-treated with non-agonist ligands and doxorubicin, while the control cells continue to be sensitized. As an alternative approach, we performed shRNA mediated knockdown of TP53 from A549 cells. Lentiviral transduction of A549 cells with a shRNA directed against p53 resulted in significantly decreased p53 accumulation. Control cells infected with a scrambled shRNA continue to show increased γ -H2AX when treated with the non-agonist ligand SR10171 and doxorubicin. Contrastingly, A549 cells that have been depleted of p53 show no increased γ -H2AX accumulation upon co-treatment with doxorubicin and SR10171 (Appendix Figure 1D). These data suggest that p53 is required for the ability of non-agonist PPAR γ ligands to sensitize cells to genotoxic agents.

For this aim, we are still planning to analyze human samples for the gene set changes as described above, and are in the process of obtaining HRPO approval to do so. I have applied for a 1 year no cost extension due to unforeseen difficulties in doing so.

As an alternative approach to looking at human data, we queried whether expression of the combined gene set correlated with the outcomes of patients treated with chemotherapy in publicly available gene expression datasets. Patients in the Director's Challenge Consortium who received adjuvant chemotherapy (n=90) and the UT Lung SPORE cohort (n=49), two of the largest cohorts of lung cancer patients receiving adjuvant chemotherapy with available gene expression data, were classified based on their expression of the genes in the signature. Overexpression (as defined by expression greater than the mean of the expression across the cohort) of the upregulated genes and underexpression (expression less than the mean) of the downregulated genes was used to create a signature score by which patients were classified. Kaplan Meier analysis of overall survival in these two combined cohorts showed that patients with greater than median signature score had a trend towards better survival than those who did not express the signature ($p=0.097$) (Appendix Figure 1E.) Analysis of these studies separately showed a similar trend when analyzed as a individually as well ($p=0.1$ and $p=0.34$ Figure S3C.)

To examine a separate cohort of patients, we used the KMplot online tool (www.kmplotter.com) to examine whether this gene set was associated with chemotherapy outcome in other cohorts. A multigene classifier dividing groups based

on mean gene expression (with equal weighting of all the genes and with negative weighting of downregulated genes) showed that patients with lung cancer treated with chemotherapy showed a trend for improved outcomes with a hazard ratio of 0.27 ($p=0.0507$) (Appendix Figure 1F), although the analysis was limited by a small number of patients ($n=34$). Of course, all of these analyses are limited due to the mixed clinical and pathologic features of these cohorts of patients. However, these data suggest that low expression of the downregulated genes and high expression of the upregulated genes is associated with improved outcomes among patients receiving systemic chemotherapy.

Aim 2 / Major task 1: Immunoprecipitation of PPAR γ from cells treated with and without carboplatinum and non-agonist PPAR γ ligands.

One of the goals of this project was to use an unbiased approach to purify a complex of phosphorylated and non-phosphorylated PPAR γ in the presence and absence of carboplatin. We were unable to generate enough PPAR γ protein from fibroblasts from the knock-in mice, which was our initial preferred approach. We have established conditions for immunoprecipitation of PPAR γ using H460 cells, which do not express significant amounts of PPAR γ , which are then transduced with retrovirus expressing either wild type PPAR γ or S273A mutant PPAR γ (Appendix Figure 2A.) With this system for purification, we are working with our mass spectrometry colleagues to identify potential binding partners.

Aim2/ Major task 2: Assess protein complex members for function in response to carboplatin.

Although we are still working on our unbiased mass spectrometry approach to yield results, I had also proposed targeted immunoprecipitation experiments to explore potential candidates that may play a functional role in the sensitization of lung cancer cells to non-agonist ligands. I previously presented data regarding the association of p53 with PPAR γ , which has been confirmed to have a functional role as described above. Meanwhile, we have also shown that PPAR γ differentially associates with other important proteins in the DNA damage response. We performed immunoprecipitation of PPAR γ from nuclear extracts of WT or mutant fibroblasts in the presence and absence of carboplatin. Immunoblotting for γ -H2AX demonstrates that the wild type PPAR γ associates less strongly with phosphorylated PPAR γ than with the unphosphorylated S273A form. This was also the case in nuclear extracts of A549 cells treated with SR1664, where phosphorylation is inhibited (Appendix Figure 2B). These data suggest that upon DNA damage, unphosphorylated PPAR γ binds to γ -H2AX, which then dissociates upon S273 phosphorylation. The exact time course of this interaction will need to be explored more fully. We are also examining the potential functional consequences of this interaction using a similar loss of function approach as described above.

Using a similar approach, we have also found that the DNA damage associated protein BRCA1 is associated with PPAR γ (Appendix Figure 2C.) Immunoprecipitation of PPAR γ from nuclear extracts of WT and knock in mutant fibroblasts followed by analysis by Western blotting, shows that BRCA1 associates with PPAR γ S273A more than the

WT protein both before and after carboplatin treatment. Again, we are in the process of examining whether this interaction is functionally important using a loss of function approach as described above.

AIM 3 / Major task 1 and 2: Mouse studies with KRAS mouse and mouse studies with KRAS/LKB1 mice.

We have had some delays in cohort assembly and tumor induction in our mouse model, and thus have not analyzed the data from these experiments. I have applied for a one year no cost extension to continue these experiments and I anticipate obtaining this data during this period.

Training and Professional Development Opportunities

This project has provided a number of opportunities for training. I have had the opportunity to attend weekly clinical conferences as well as monthly research conferences. I was also able to present my work at the Spiegelman Lab group meeting, and get feedback from a group of scientists with expertise in a diverse group of fields. I met with Dr. Spiegelman, my mentor, at least twice a month to review data and discuss scientific issues and directions. An abstract based on this work was accepted as a poster presentation at the Keystone Symposium Conference: New Frontiers in Understanding Tumor Metabolism last year.

Dissemination of Results

We have a manuscript that we have submitted for publication. The results are still pending at this time.

Plans for Next Year

In the next year, I am planning to analyze the clinical samples after obtaining IRB approval. We should also obtain our unbiased analysis of proteins interacting with PPAR γ . We will also have analyzed the importance of the γ -H2AX interaction and BRCA1 interactions described above. Finally, we should have data from our mouse experiments with the KRAS inducible tumors in the PPAR γ WT and S273A backgrounds. These goals should complete the experiments outlined in the grant.

IMPACT

We are in the process of publishing a manuscript that covers the experiments outlined in this grant. We anticipate that there will be interest in the lung cancer community as well as in the cancer community as a whole. For patients who lack a oncogenic driver mutation and who do not respond to immunotherapy, traditional cytotoxic chemotherapy remains the standard of care. Thus, the availability of a relatively non-toxic agent that can be added to standard of care therapy would be a big advance. Furthermore, we are hopeful that by identifying biomarkers of efficacy (e.g. the gene sets identified in Aim1, as well as the presence of both PPAR γ and P53), we can specifically identify a group of patients who are most likely to benefit from combination therapy and thus maximize the likelihood of success in early phase studies.

This work may have implications for other disciplines as well. By showing the ability of these drugs to modulate PPAR γ activity in lung cancer, we are hopeful that other cancer types where PPAR γ is expressed (e.g. breast cancer, endometrial cancer, ovarian cancer,) may also be potential candidates for combination therapy. We have preliminary data in breast cancer cells that these drugs are able to sensitize them to DNA damaging agents. Furthermore, we hope that demonstration of the relative safety of these non-agonist ligands in cancer may propel them to be explored as anti-diabetic agents for metabolic disease as well.

Our data describing the physical interaction between p53 and PPAR γ is novel, and may suggest that PPAR γ has a role in modifying p53 function in other tissues. It has been shown that p53 plays an important role in metabolism and adipose tissue function, and this may be modulated by PPAR γ expression as well.

At this point, I do not have anything to report regarding potential impacts on technology transfer or on society beyond science and technology as a whole.

CHANGES/PROBLEMS

Changes in approach: Nothing to report

Actual or anticipated problems or delays: We have had delays in a few areas which have pushed back our timeline from the SOW. For Aim 1, I am still in the process of obtaining HRPO approval for the clinical samples, which has delayed completion. However, given that we have the gene set information already identified, analysis of these samples will still be able to be completed within the time of the no-cost extension.

For Aim 2, it took longer than anticipated to find appropriate conditions to immunoprecipitate an amount of PPAR γ protein from these cells sufficient for mass spectrometric analysis. We should obtain this data soon, which will expand our search for interacting partners. Meanwhile, we have used our alternative candidate gene approach to identify 3 binding partners of PPAR γ : p53, γ -H2AX, and BRCA1.

For Aim 3, we have had delays in cohort assembly and tumor induction, which has taken longer than outlined in the SOW. However, I believe we will be able to complete analysis of these results within the no-cost extension period.

Changes that had impact on expenditures: We have come in under budget as most of the experiments outlined above involve overhead and supplies that are paid via my mentor's grant and institutional funding.

Changes in use of human/animals: Nothing to report

PRODUCTS

Publications, conference papers, and presentations.

Poster Presentation: Melin J. Khandekar, Alex S. Banks, Dina Laznik-Bogoslavski, James P. White, Jang H. Choi, Kwok-kin Wong, Ted Kamenecka, Patrick R. Griffin, Bruce M. Spiegelman. "Non-agonist PPAR-gamma ligands sensitize cancer cells to the effects of cytotoxic chemotherapy by inhibiting phosphorylation of Serine 273 and modulating the response to DNA damage." New Frontiers in Understanding Tumor Metabolism, Banff, Canada. February 2016

Invited Oral Presentation: Selective modulation of PPAR-gamma influences the response of cells to DNA damage and potentiates cytotoxic therapy. Obesity and Cancer Working Group Meeting. Memorial Sloan Kettering Cancer Center. New York, NY. March 21, 2016.

Manuscript: Non-agonist PPAR-gamma ligands sensitize cancer cells to the effects of cytotoxic chemotherapy and modulate the response to DNA damage. Melin J. Khandekar, Alexander S. Banks, Dina Laznik-Bogoslavski, James P. White, Jang H. Choi, Lawrence Kazak, James C. Lo, Paul Cohen, Kwok-kin Wong, Ted Kamenecka, Patrick R. Griffin, Bruce M. Spiegelman (Submitted)

PARTICIPANTS

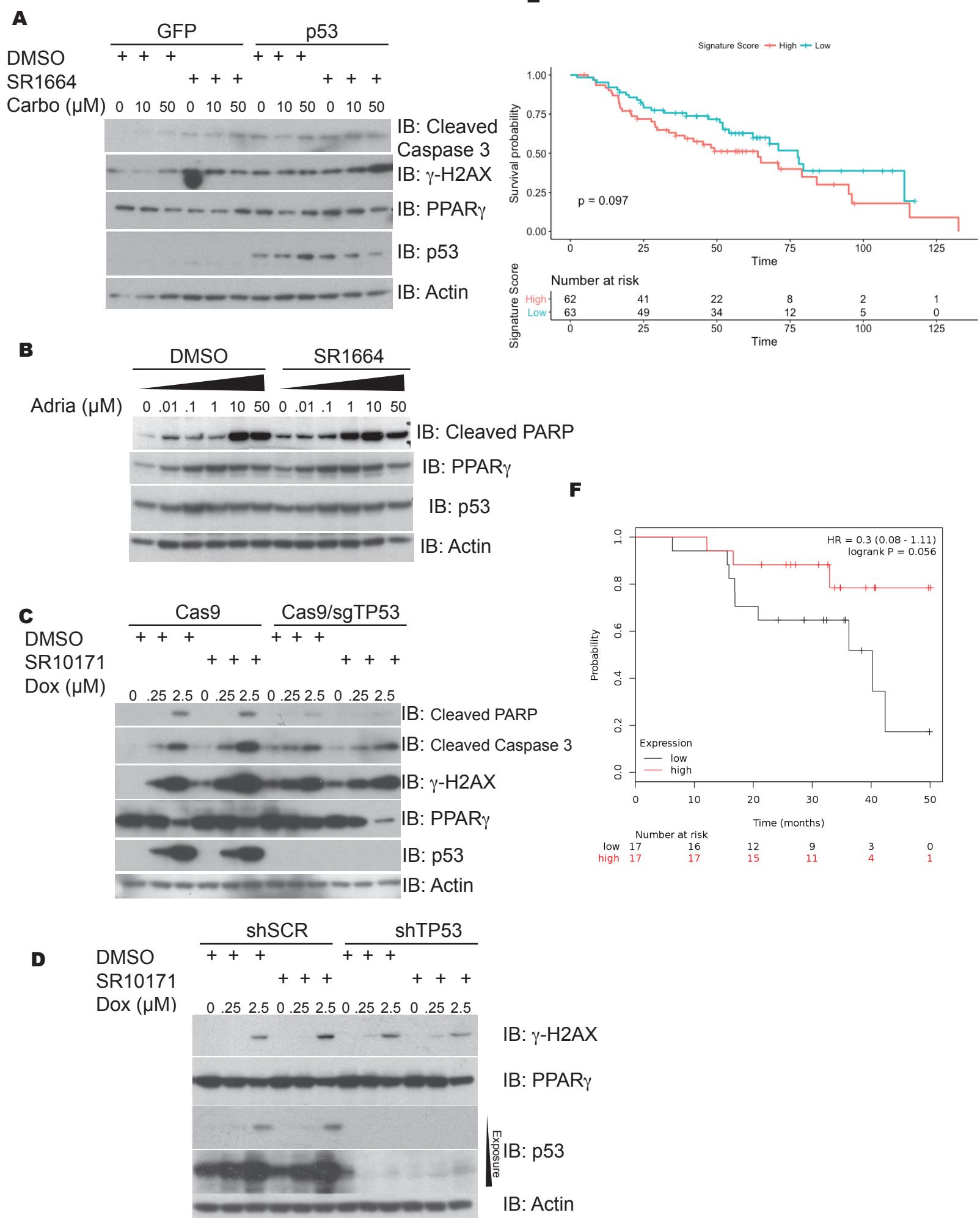
Name:	Melin Khandekar
Role:	PI
Nearest Person Month Worked	6 months
Contribution:	Performed all work described above
Funding Support	No change

Name:	Bruce Spiegelman
Role:	Mentor
Nearest Person Month Worked	NA
Contribution:	Advised PI regarding experimental design, and analysis of experiments
Funding Support	No change

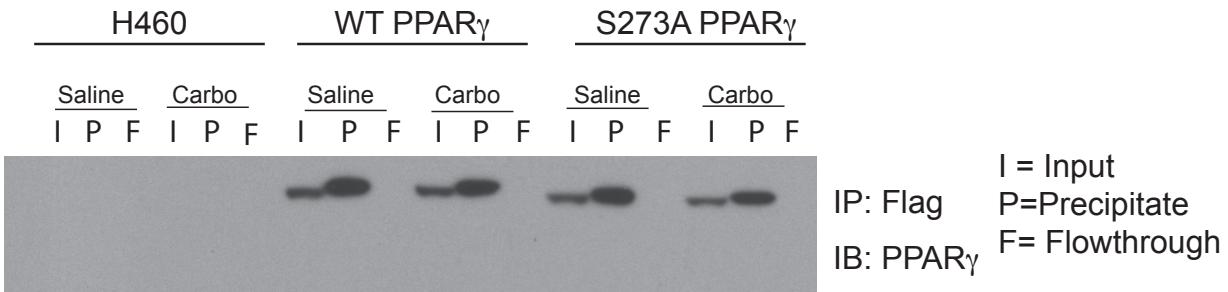
Other Partner Organizations

Name: Dana Farber Cancer institute
Location: Boston, MA
Contribution: Facilities and collaboration with mentor's laboratory staff

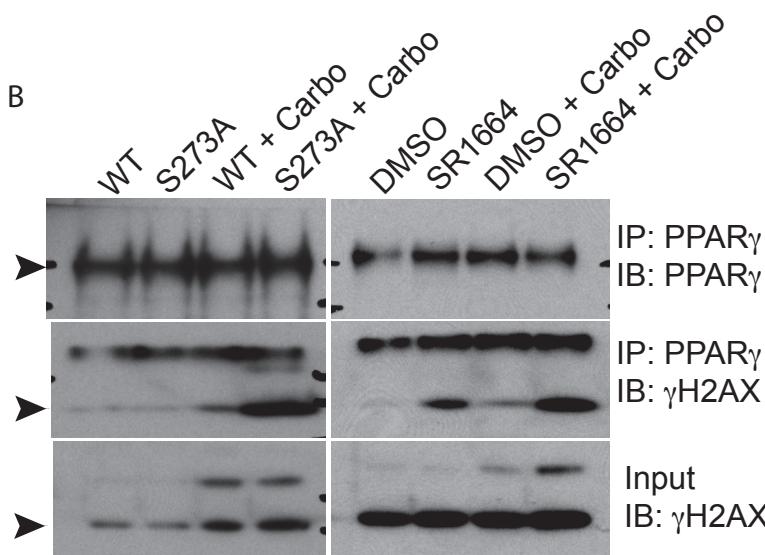
Name: The Scripps Research Institute Florida
Location: Jupiter, FL
Contribution: In kind support (reagents) and collaboration regarding non-agonist ligand dosing



A



B



C

